A NOVEL STRATEGY TO CONTROL AND PREVENT NOROVIRUS GASTROENTERITIS

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ABSTRACT

Norovirus (NV) gastroenteritis is an important disease of all ages and populations, including the military. The disease is difficult to control due to its widespread nature and lack of a treatment. NVs recently have been found to recognize human histo-blood group antigens (HBGAs) as receptors. The human HBGA system is highly polymorphic; NVs also are genetically diverse. This presentation summarizes our studies on the unique interaction of NVs with human host and the discovery of diversified receptor binding patterns of NVs. We also developed a saliva-based enzyme immune assay (EIA) to screen for inhibitors against NVs binding to receptors using a compound library. We discovered over a dozen compounds that potentially could be developed into antivirals against NVs. Human NVs still cannot be cultivated and infect animals. Our studies have significantly advanced the field of NV research. Further characterization of the NV/host interaction and application of new knowledge gained from these studies would result in development of new strategy to control and prevent NV gastroenteritis.

INTRODUCTION

Norovirus (NV) gastroenteritis is an important disease in both developed and developing countries. The virus is highly contagious and commonly causes large outbreaks in a variety of settings, such as schools, child care centers, hospitals, restaurants, nursing homes for the elderly, cruise ships and the military. The importance of the disease in the military has been particularly highlighted by the high prevalence of NV gastroenteritis detected among US and British troops in recent military operations in Kuwait (Operation Desert Storm, 1991), Afghanistan (Operation Enduring Freedom, 2002) and Iraq (Operation Iraqi Freedom, 2003). Large outbreaks of NV gastroenteritis also commonly occur in the military during peace time, such as among crews of large battle ships during deployment and soldiers in recruit and training camps. The disease is difficult to control due to its wide spread nature and a lack of antiviral or vaccine against NVs. Since the 9/11 tragedy, NVs have been listed as category B priority agent in the NIH/CDC Biodefense Program.

NVs are difficult to study due their wide genetic and antigenic variation and the lack of cell culture or animal models. The cloning of NVs have resulted in expression of the viral capsid protein in baculovirus which spontaneously forms virus-like particles (VLPs) that are morphologically and antigenically similar to the authentic viruses (Jiang; Wang et al. 1992). In the past decade we have accumulated recombinant VLPs for over dozen of NV strains representing different genetic types and these VLPs provided us excellent tools to study the immunology, pathogenesis, host/pathogen interaction, and receptors of NVs.

Using the recombinant VLPs, we first reported that the prototype Norwalk virus (first found in Norwalk, Ohio, 1969) recognizes the carbohydrate receptors of the human histo-blood group antigens (HBGAs) (Marionneau; Ruvoen et al. 2002). The link between this pathogen/host interaction with clinical infection and illness was later confirmed by human volunteer studies (Lindesmith; Moe et al. 2003). The Norwalk virus recognizes only the H antigens (1,2 linked fucose epitope) of secretors but not the HBGAs of non-secretors. In a volunteer study of 55 secretors and 22 non-secretors challenged with the Norwalk virus, 35 of the 55 secretors, but none of the 22 non-secretors, developed clinical disease or were infected by the virus (Lindesmith; Moe et al. 2003). This result provided the first direct evidence to support the hypothesis proposed in the early1970s of a genetic factor being involved in NV infection.

HBGAs are complex carbohydrates present at the outermost part of N- or O-linked glycans of many glycoproteins and glycolipids. These complex carbohydrates are abundant on the surfaces of red blood cells and mucosal epithelium of the respiratory, genitourinary, and digestive tracts. They are also present as free oligosaccharides in biologic fluids, such as saliva, intestinal contents, milk, and blood. The synthesis pathways of these HBGAs are controlled genetically by the ABO, Lewis and secretor gene families of the hosts. A number of microorganisms (*Helicobacter pylori*, *Campylobacter*, uropathogenic *Escherichia coli*, *Vibrio cholerae*, *Candida*, etc.) have been reported to recognize the HBGAs as their receptors. NV is the first viral pathogen in the list.

Human NVs are also genetically diverse. Since the cloning of the prototype Norwalk virus in 1990, many morphologically similar Norwalk-like viruses have been

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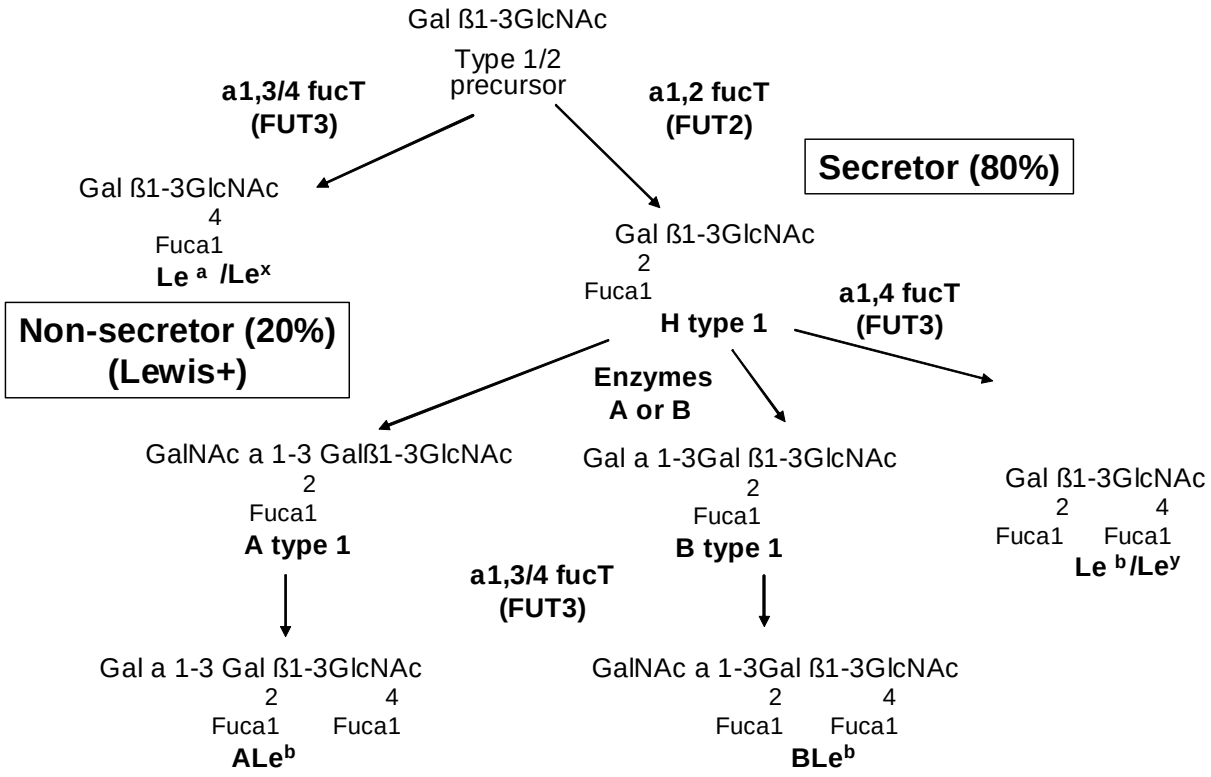

Figure 1. Biosynthesis pathways of HBGAs based on type 1 molecules.

cloned. Genetic analysis has shown that all of these viruses, now called Noroviruses, are genetically related. Over 25 genetic clusters within five genogroups of human NVs have been described (Zheng; Ando et al. 2006). Following the description of the Norwalk virus receptor, we expanded our research to examine many other NV strains representing different genetic types and we have found that NVs are diverse in their recognition of HBGA receptors. This presentation summarizes these findings. We also developed a screening method based on the specificity of NV/receptor interaction and identified over a dozen of compounds that are potentially developed into antivirals against NV gastroenteritis.

METHODS

Virus-like particles (VLPs) of NVs. VLPs of fourteen strains representing thirteen genetic clusters of Norovirus produced from baculovirus were used in this study, including genogroup I: Boxer (AF538679), C59 (AF435807), Norwalk virus (M87661), Desert Shield virus (DSV; U044469), VA115 (AY038598); and genogroup II: BUDS (AY660568), Grimsby (GrV; AJ004864), Hawaii (HV; U07611), Mexico (MxV; U22498), MOH (AF397156), Parris Island (PiV; AY652979), VA387 (AY038600), VA207 (AY038599), and Operation Iraq Freedom 031998 (OIF, AY675554). Among them, Norwalk, VA387, MOH and VA207 represent the 4 previously published receptor binding patterns (Huang; Farkas et al. 2003). The procedures of production of Norovirus VLPs in insect cell culture have been published previously (Jiang; Wang et al. 1992: Jiang; Matson et al. 1995: Jiang; Zhong et al. 2002). VLPs were partially purified by sucrose gradient centrifugation and stored at -70°C. Protein concentrations were determined by measuring OD_{280} and by comparison with a bovine serum albumin standard in SDS-polyacrylamide gel (Tan; Huang et al. 2003).

Binding of NV VLPs to saliva samples. Saliva samples from healthy volunteers were collected under an approval of human subject research protocol by the Institutional Review Board at the Cincinnati Children's Hospital Medical Center. The saliva-binding assays were performed as described previously (Huang; Farkas et al. 2003). Briefly, microtiter plates (Dynex Immulon, Dynatech, Franklin, MA) were coated with saliva at a dilution of 1: 1,000 to 5,000 in phosphate buffer saline (PBS, pH 7.4). To avoid potential NV specific antibodies in the saliva that may interfere in the receptor binding assays, saliva samples were boiled before being used in the assays. After removing the unbound saliva and blocking with 5% dried milk (Blotto), baculovirus-expressed NV VLPs at a concentration of 0.4-1.0 μg/mL in PBS were added. The bound VLPs were detected using a

pooled guinea pig anti-NV antiserum (dilution of 1: 3,000), followed by addition of horseradish peroxidase (HRP)-conjugated goat anti-guinea pig IgG (ICN, Aurora, OH). The pooled guinea pig anti-NV antiserum was a mixture of hyper-immune sera of 3 groups of guinea pigs cross-immunized with three sets of recombinant NV capsid antigens: set 1, Norwalk virus, C59 and VA115; set 2, VA387, Mx, GrV and HV and set 3, VA207 and MOH. The pooled guinea pig anti-NV antiserum recognized recombined capsid proteins of all 14 NV strains studied.

Binding of VLPs to synthetic oligosaccharides containing human HBGA epitopes. Microtiter plates were coated with synthetic oligosaccharide-BSA conjugates containing human HBGA epitopes at a concentration of 20 μg/mL at 4°C overnight. Oligosaccharide-PAA-biotin conjugates (2 μg/mL) were coated to microtiter plates by means of streptavidin. After blocking with 5% Blotto, NV VLPs were added at 0.4-1.0 μg/mL. The captured VLPs were detected by the same procedures described above. Oligosaccharides conjugated with two types of carriers were used in this study, including polyacrylamide (PAA)-biotin conjugates: H type 3-, type A- and type B-disaccharides; Le^a-, Le^x-, H type 1-, H type 2-, type A- and type B-trisaccharides; Le^b-, Le^y-, sLe^a-, and sLe^x-tetrasaccharides (GlycoTech Corporation, Rockville, MD); and bovine serum albumin (BSA) conjugates: A-trisaccharide-BSA {[GalNAc α 1-3 (Fuc α 1-2) Gal β-O-spacer]$_n$-BSA]} and B-trisaccharide-BSA {[Gal α 1-3 (Fuc α 1-2) Gal β-O-spacer]$_n$-BSA}, both BSA conjugates containing a 5-atom spacer (Glycorex AB, Lund Sweden). Additional A-trisaccharide-BSA and B-trisaccharide-BSA, with a 20- and a 3-atom spacer respectively also were used (V-Labs, Inc., Covington, LA).

Compound library. The compound library tested in this study was "The Diversity Screening Set" (Timtec Inc., Newark, DE) which is a collection of diverse, highly pure, rationally selected, drug-like small molecule compounds ranging from 200 to 850 Daltons. A database of the compound library is available at http://www.timtec.com. The compounds were received as powders which were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM and stored at -20^{o}C before use. DMSO concentrations in the reaction mixture were managed to be never higher than 0.5% (vol/vol).

A saliva-based enzyme immune assay (EIA) to screen compounds for blocking NV binding to human HBGA receptors. This EIA was developed to measure the inhibitory activity of compounds against VA387 VLPs binding to the A antigen. Standard 96-well microtiter plates (Dynex Immulon; Dynatech, Franklin, MA) were coated with the type A saliva sample described above. The baculovirus-expressed VA387 VLP were incubated with or without compounds at 37 °C for 30 min, then the reaction mixture was added to the microtiter plates coated with the type A saliva. The bound VLPs were detected following the same procedures described above. The hits from the primary screening were retested with serial dilutions of the compounds (from ~0.1 to ~100 μM), and the blocking activity of each compound was expressed as 50% effective concentration (EC_{50}) by a comparison of the signals in wells with the compounds with that of the negative control wells without compound (column 1) minus the background noise (the blank control wells in column 8 that contain all components except compounds and VLPs).

Following the primary screening, compounds with significant blocking activities against VA387 binding to the A antigen were further tested for their inhibitory activity of: 1) VA387 binding to types B and H antigens, 2) Norwalk virus binding to the A and H antigens, 3) MOH binding to the A and B antigens, and 4) VA207 binding to the Lewis antigens. The same format and condition of the assays described above, except variable concentrations of VLPs (100 to 500 ng/ml), were used. The is because the receptor binding affinity of individual strains varies (Huang; Farkas et al. 2003) and based on titration results, we selected a concentration to give an OD_{450} = ~1 in the wells without compounds for each strain.

RESULTS

NVs are diverse in recognizing human HBGA receptors. Using a panel of saliva representing different blood types, we identified (Huang; Farkas et al. 2005) 7 distinct receptor-binding patterns among 14 NVs characterized (Table 1). The 7 binding patterns covered all 3 major HBGA families (the ABO, secretor, and Lewis families) with each binding pattern reacted with a single or a combination of different HBGA types. In a separate study, Harrington and co-workers reported another binding pattern (Harrington; Lindesmith et al. 2002). Thus, at least 8 binding patterns of NVs with HBGAs exist. Noteworthy, 2 other strains (VA115 and DSV) do not bind to any tested human HBGAs (Huang; Farkas et al. 2005), implying that molecules other than HBGAs might serve as receptors for these two strains.

When synthetic oligosaccharide conjugates representing different HBGAs were used in binding assays, most of the deduced binding epitopes have been confirmed (Huang; Farkas et al. 2005). However, the oligosaccharide-binding assays also revealed some binding activities that were not observed in the saliva-based assays (Huang; Farkas et al. 2005). These extra binding activities are dose-dependent and reproducible. These discrepancies indicate subtle differences between the native antigens in saliva and the synthetic oligosaccharides. Since saliva contains the authentic antigens *in vivo*, the saliva-based assays probably have more biological relevance in term of host specificity. However, synthetic antigens might stand for some subtypes of HBGAs present on the intestinal epithelium but absent in saliva.

Further studies using HBGAs from human intestine may help to clarify this issue.

Strains in the Lewis binding group do not recognize the A and the B epitopes; instead, the presence of the A/B epitopes may have a negative effect (epitope masking) on the binding to the Lewis and H epitopes. This is consistent with the relative lower binding activities of those strains with types A and B saliva compared with saliva from non-secretors and type O secretors (Table 1 and Figure 2), and the binding to the A and B saliva could result from precursor molecules such as Lewis$^{a/x}$ or Lewis$^{b/y}$ before being mature to A and B antigens, respectively (Figure 1).

Table 1. HBGA receptor binding patterns of NVs based on saliva- and oligosaccharide conjugate-EIAs.

Binding groups	Binding patterns	Representing strains	Predicted reactive epitopes of HBGAs			
			Non-secretor	Secretor		
			Le$^{a/x}$	H	A	B
A/B	1	VA387	-	+++	++++	++++
	2	Norwalk	-	+++	++++	-
	3	MxV	-	+	+++	+++
	4	MOH	-	-	++++	++++
	5*	SMV	-	-	-	+++
Lewis	6	VA207	+++	++	+	+/-
	7	Boxer	++	+++	+	+/-
	8	OIF	++	+/-	-	-
		DSV, VA115	-	-	-	-

* SMV, Snow Mountain virus, the receptor binding pattern was determined by Harrington et al (Harrington; Vinje et al. 2004).

A model of NV-HBGA receptor interaction. In summary of the binding results from the saliva- and the synthetic oligosaccharide-based assays (Harrington; Vinje et al. 2004: Harrington; Lindesmith et al. 2002: Huang; Farkas et al. 2003: Huang; Farkas et al. 2005), the 8 known binding patterns can be sorted into two groups: the A/B and the Lewis (non-secretor) binding groups (Huang; Farkas et al. 2005). All strains in the A/B binding group bound to types A, and/or B, and/or O saliva of secretors but not to saliva of non-secretors. All strains in the Lewis binding group bound to HBGAs of non-secretors and type O secretors with weak or no binding to the types A and B secretors. To elucidate the relationship of the two binding groups, a model of NV-HBGA interaction has been proposed (Huang; Farkas et al. 2005) (Figure 2). This model assumes that the capsid of each strain has one binding interface that can accommodate a maximum of two epitopes of the three major HBGAs (A/B, H, or Lewis). Each epitope can bind to the interface independently, whereas involvement of both epitopes simultaneously in binding may broaden the host specificity of a strain. Thus, all strains in the A/B binding group have a common site for the A/B epitopes, and some strains have an additional site for the H epitopes. In the Lewis binding group, all strains have a binding site for the Lewis epitopes (Le^a and Le^x) and some strains have an additional site for the H epitopes.

Genetic relationship of NV receptor-binding specificities. The fact that the two binding groups mutually exclude the A/B or the Lewis epitopes indicates that the two groups may represent two genetic lineages in the co-evolution with the human host. Unfortunately, no clear segregation of the two binding groups with the genogroups of NVs could be seen when the entire capsid sequences were analyzed, although strains with the same or closely related binding patterns are clustered (Huang; Farkas et al. 2005). These data suggest that the binding specificity may be determined by small regions of the capsid protein, possibly just a few scattered amino acids that are involved in the formation of the binding interface of the viral capsids. Thus, the routine phylogenetic analysis may not be the right way to detect such minor genetic variation in relationship with the binding patterns between the two binding groups. On the other hand, other factors, such as the acquired host immunity, may also play a role in the evolution of NVs, which can further complicate the situation.

The above data indicate that NVs are among hose highly adaptable species of human pathogens. Although all currently known NV strains are predominantly mild, non-life-threatening pathogens that would not tend to cause a major selection pressure on the human host, it should not exclude the possibility that a highly virulent NV strain might have once existed in the past and/or may occur in the future. The RHDV is believed to be an emerging virulent strain that had wiped-out almost the entire rabbit colonies when it first appeared in China and European countries in the 1980s (Cooke 2002: Marchandeau; Le Gall-Recule et al. 2005: Sanchez-Campos; Alvarez et al. 2004: White; Norman et al. 2002: White; Trout et al. 2004: Wirblich; Meyers et al. 1994). The high adaptation feature of NVs suggests there may also be an animal reservoir of human NVs. The increasing evidence of animal NVs causing gastroenteritis in different domestic animals should keep us alert for such a possibility. The scenario of HIV, influenza virus, and the recently reported panic disease of SARS have set up examples that pathogens of animal origin can infect humans under certain circumstances. Thus, the study of the origin and evolution of NVs will provide useful information for the overall understanding of interaction between pathogens and their human hosts.

Screening a compound library for inhibitor against NVs binding to HBGA receptors. Following the description of different receptor binding patterns, we used the saliva-based

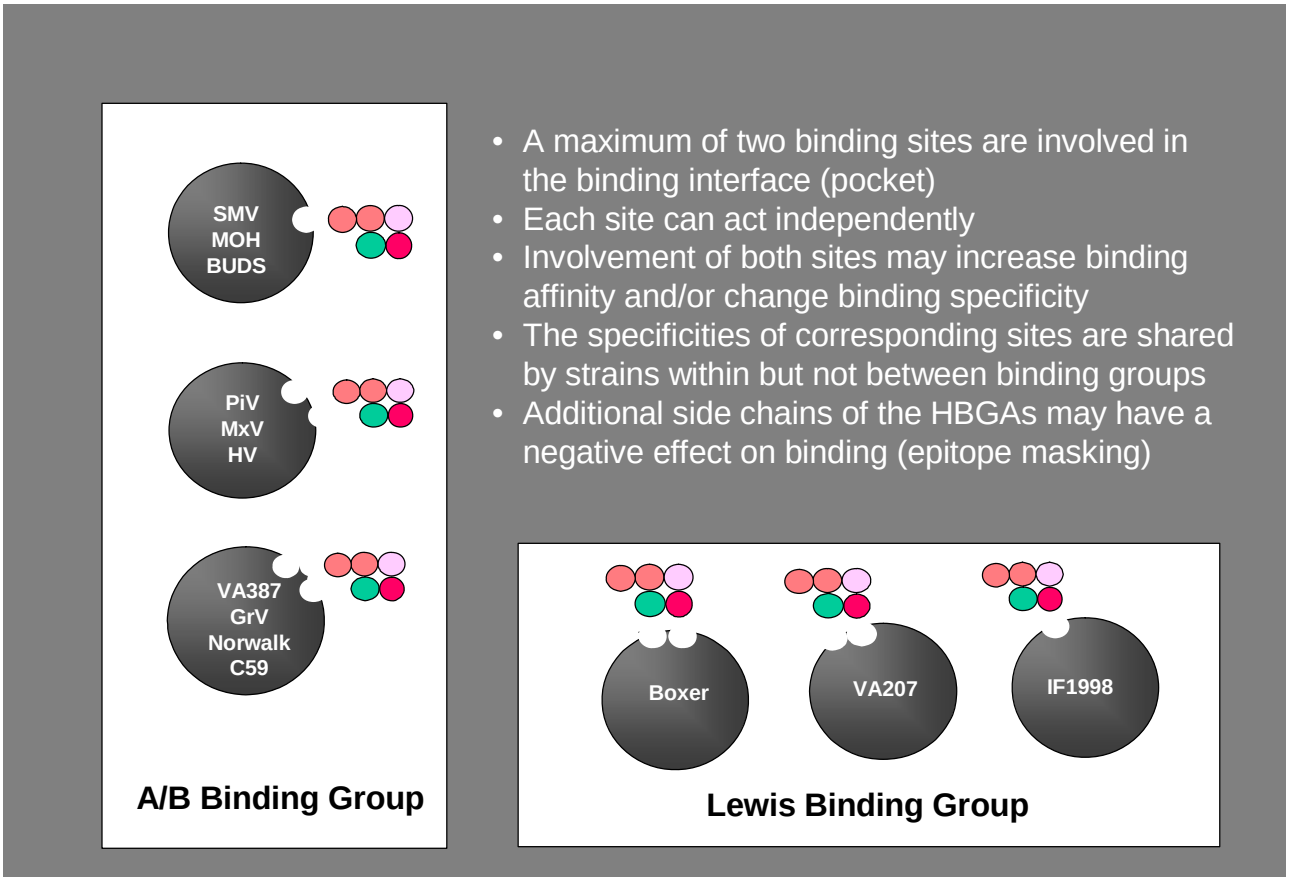

Figure 2. A model of the Norovirus/HBGA interaction. This model is developed based on the classification of two binding groups of Norovirus to human HBGAs, the "A/B binding group" and "the Lewis binding group". The five-circle structure shown on the top-right of the figure represents an pentasaccharide as the final products (ALe^b or BLe^b) of the human HBGAs. Thirteen Norovirus strains are indicated according to their relative interaction levels with the A/B, H and Lewis epitopes (Table 1). Strain SMV was reported to recognize the type B antigen (Harrington; Lindesmith et al. 2002). The potential binding sites for each of the three HBGA epitopes on the capsid are indicated. The specificities of individual binding domains may be different for the A and B epitopes, in the cases of Norwalk virus, C59, BUDS, and SMV, but the same shape of the binding sites was used for convenience.

EIAs to screen a compound library for inhibition or competition of NV/receptors interaction. Our hypothesis is that if a compound specifically blocks NVs binding to their receptors, this compound could be further developed into an antiviral to treat or prevent NV infection or illness. Among 5000 compounds screened so far, 153 compounds exhibited more than 50% inhibitory effects of VA387 (GII/4) binding to A saliva at a concentration of ~100 μM and 15 of the 153 compounds revealed strong inhibition with a 50% effective concentration (EC_{50}) value less than 15 μM. Ten and eleven of the 15 compounds also revealed inhibition against the binding of VA387 to the B and H antigens respectively. Seven and six of the 15 compounds also blocked Norwalk (GI/1) VLPs binding to the A and H antigens, respectively. One compound significantly inhibited MOH (GII/5) binding to the A and B antigens, but none revealed inhibitory effect on a Lewis binding strain (VA207, GII/9) to the Lewis antigens (Table 2).

TABLE 2. Inhibitory effects of the 15 lead compounds against binding of NVs to the corresponding HBGAs[a]

Compound	EC_{50} (μM)[b]								CC_{50} (μM)[c]
	rVA387			rNorwalk		rVA207	rMOH		
	A	B	H	A	H	Le	A	B	
1	2.2	5.8	6.8	15.6	18.9	>100	>100	>100	158.0
2	4.8	18.6	11.9	22.9	24.5	>100	16.5	13.3	78.6
3	7.8	>100	>100	>100	>100	>100	>100	>100	82.7
4	8.1	13.1	14.2	14.1	22.6	>100	>100	>100	112.6
5	9.1	13.5	7.2	18.2	36.3	>100	>100	>100	46.7
6	9.3	44.6	41.1	>100	>100	>100	>100	>100	52.7
7	9.6	26.0	7.6	>100	>100	>100	>100	>100	323.6
8	10.9	>100	>100	>100	>100	>100	>100	>100	157.3
9	11.7	>100	47.5	>100	>100	>100	>100	>100	40.3
10	11.8	7.9	7.5	35.0	19.8	>100	>100	>100	220.3
11	12.8	35.3	26.1	>100	>100	>100	>100	>100	262.8
12	12.8	32.5	28.2	53.0	47.8	>100	>100	>100	180.2
13	13.6	49.5	48.1	>100	>100	>100	>100	>100	70.6
14	13.7	>100	>100	>100	>100	>100	>100	>100	12.3
15	14.1	>100	>100	22.5	>100	>100	>100	>100	7.6

[a] Experiments were repeated three times, and the data in the table show the average EC_{50} values.
[b] EC_{50}: effective concentration to achieve 50% inhibition of various Noroviruses binding to the corresponding HBGAs.
[c] CC_{50}: the concentration of a compound to cause 50% cell death. Experiments were repeated three times, and the data in the table show the average CC_{50} values.

CONCLUSIONS

In conclusion, the finding of NV receptor has opened a new strategy to design antivirals against NVs. The saliva-based EIA is a high throughput method for large-scale screening of compound libraries. Further studies to characterize the NV receptors, to screen the compounds library using other NV VLPs representing additional receptor binding patterns, as well as to characterize the newly identified effective compounds

for higher affinity and specificity by modification and re-synthesis of the compounds are necessary.

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